A Modified Colorimetric Method for Phytic Acid Analysis in Soybean

Y. Gao, C. Shang, M. A. Saghai Maroof,* R. M. Biyashev, E. A. Grabau, P. Kwanyuen, J. W. Burton, and G. R. Buss

ABSTRACT

A quantitative, reproducible, and efficient phytic acid assay procedure is needed to screen breeding populations and support genetic studies in soybeans [*Glycine max* (L.) Merr.]. The objective of this study was to modify the colorimetric Wade reagent method and compare the accuracy and applicability of this new method in determining seed phytic acid content in soybean with three well-established phytic acid assay methods: anion exchange column (AEC), high-performance liquid chromatography (HPLC), and ^{31}P nuclear magnetic resonance (NMR). The CV for repeated measurements of a low phytic acid soybean mutant, CX1834-1-6, ranged from 1.8 to 4.2% (n = 5), indicating the results were precise and reproducible. Phytic acid content of 42 soybean genotypes as determined by this method showed a correlation of 93.7 to 96.6% with the measurements by AEC, HPLC, and NMR. According to analysis of covariance, using inorganic P content as a predictor, phytic acid P content in a given sample analyzed by the four assay methods can be estimated with four linear regression models at the α = 0.01 level. Compared with HPLC, AEC, and ^{31}P NMR, this modified colorimetric method is simpler and less expensive for assaying a large number of samples, allowing its effective application in breeding and genetic studies of low phytic acid soybean.

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Abbreviations: AEC, anion exchange column; ANCOVA, analysis of covariance; dd, distilled-deionized; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; ICP, inductively coupled plasma emission spectroscopy; IP, inositol phosphate; NMR, nuclear magnetic resonance; PA, phytic acid; Pi, inorganic phosphorus; QTL, quantitative trait loci.

A simple, reproducible, and efficient phytic acid (PA) assay is required to conduct genetic studies and breeding of low PA soybean [*Glycine max* (L.) Merr.]. Currently, an indirect assay based on the inverse relationship between inorganic phosphorus (Pi) and PA (Chen et al., 1956; Raboy et al., 2000) has been widely used to estimate PA levels in soybean seed samples. Applications of such indirect PA estimations can be found in previous isolation of low PA soybean mutants (Wilcox et al., 2000), in a study of genetic inheritance of the low PA soybean mutant CX1834-1-6 (Oltmans et al., 2004), and in genetic mapping of loci associated with low PA genes in CX1834-1-2 (Walker et al., 2006). This indirect assay has been shown useful for detecting the change in seed PA-P associated with little change in seed total P. However, screening low PA soybean lines from populations with diverse backgrounds and different total P content requires accurate measurements of absolute levels of seed PA.

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Modified Fe(III) precipitation methods (Bowen et al., 2006; Haug and Lantzsch, 1983; Shi et al., 2003) were used to analyze PA in cereals. Because these methods indirectly determine PA content by measuring the excess Fe(III) in solution, the assay requires extra experimental efforts to remove Fe-phytate precipitates before the Fe determination (Vaintraub and Lapteva, 1988). High-performance liquid chromatography (HPLC), anion exchange column (AEC), and ^{31}P nuclear magnetic resonance (NMR) spectroscopy overcame the low accuracy, acid hydrolysis requirement, and other problems associated with the classic Fe(III) precipitation PA assay method (Reddy et al., 1989; Velickovic et al., 1999; Xu et al., 1992) and have also been used for PA assays. These methods, however, require sophisticated instruments, high maintenance, and specialized staff and are not readily adopted by plant breeders. Both HPLC and AEC methods can only analyze 10 to 30 samples per day, which makes them impractical for routine analysis involving large numbers of samples. By comparison, Latta and Eskin (1980) modified the AEC method (Harland and Oberleas, 1977) by replacing the strong-acid digestion with a simpler colorimetric method for PA-P analysis. The determination of PA content with this colorimetric method is based on the decoloration of the pink Fe^{3+}–sulfosalicylate complex (Wade reagent) by PA, which can eliminate the acid hydrolysis and associated laboratory hazards. To remove Pi interference, the Latta and Eskin (1980) procedure still needed to include the AEC step, which is time consuming. Vaintraub and Lapteva (1988) later demonstrated that by adjusting the pH of the crude extract to 2.0 to 2.5, the anion exchange purification step could be omitted by adding Wade reagent directly to acidified extracts. This modification made Vaintraub and Lapteva's method simpler. Possibly caused by some unknown matrix interferences, Vaintraub and Lapteva's method (1988) underestimated PA content (Harland and Oberleas, 1977; Latta and Eskin, 1980; Xu et al., 1992). The earlier AEC method also encountered these unknown matrix interferences, but they were amended by pretreating samples with ethylenediaminetetraacetic acid (EDTA) (Harland and Oberleas, 1977; Latta and Eskin, 1980; Xu et al., 1992).

The objectives of this study were (i) to improve the colorimetric (Wade reagent) PA assay (Vaintraub and Lapteva, 1988); (ii) to compare the improved protocol with several well-established methods, including AEC, HPLC, and ^{31}P NMR; and (iii) to determine the applicability of the modified PA method in screening of low PA soybean.

MATERIALS AND METHODS

Soybean Seed Samples

A total of 42 soybean lines with a wide range of PA content as previously determined by the modified colorimetric (Wade reagent) method were chosen for this study. The sample set included two commercial soybean cultivars Hutcheson and MN 1401, plant introductions PI 87013, PI 407162, low PA mutants CX1834-1-6 and M766 (seeds were kindly provided by J. Wilcox and V. Raboy, USDA-ARS) (Wilcox et al., 2000), four Virginia Tech experimental lines V99-5089, V99-3337, V99-8060, and MFL552, and 32 breeding lines developed from crosses of V99-5089 × CX1834-1-6 and CX1834-1-6 × V99-3337. These breeding lines were randomly selected with PA levels ranging from 6 to 22 mg g^{-1}.

Phytic Acid and Pi Analyses

A sample of 50 to 75 random seed from each soybean line were ground with a Cyclone Sample Mill grinder with a 0.5-mm mesh (UDY Corporation, Fort Collins, CO). Samples of 0.50 g of ground powder were thoroughly mixed with 10 mL of 2.4% HCl in 14-mL Falcon tubes. Sample tubes were shaken at 220 rpm for 16 h in a Model 3525 Lab-line incubator shaker (Lab-Line Instruments, Inc., Melrose Park, IL) and centrifuged at 1000 *g* (Sorvall RT6000B refrigerated centrifuge, Du Pont, Newtown, CT) at 10°C for 20 min. The crude extracts were collected for total P analysis by inductively coupled plasma emission spectroscopy (ICP) and for PA determination by four assay methods.

Modified Colorimetric (Wade Reagent) Method

The Vaintraub and Lapteva (1988) method was modified as follows: (i) the extraction time was extended to 16 h, (ii) the temperature of centrifugation was reduced to 10°C, and (iii) a matrix cleaning step was added to increase PA recovery. The details of the protocol were as follows. Crude acid extracts were transferred to 14-mL Falcon tubes containing 1 *g* NaCl. The contents were shaken at 350 rpm for 20 min to dissolve the salt and were allowed to settle at 4°C for 60 min or at −20°C for 20 min. The mixtures were centrifuged at 1000 *g* at 10°C for 20 min, and clear supernatants, hereafter referred to as the NaCl-treated supernatant, were collected for color development. This treatment precipitated matrix components that could interfere with the colorimetric reaction. One mL of the clear supernatant was diluted 25 times in a 50-mL Falcon tube by mixing with 24 mL of distilled-deionized (dd) H_2O. Three mL of this diluted sample were combined with 1 mL of modified Wade reagent (0.03% $FeCl_3 \cdot 6H_2O$ + 0.3% sulfosalicylic acid) in a 14-mL Falcon tube, thoroughly mixed on a vortex, and centrifuged at 1000 *g* at 10°C for 10 min. A series of calibration standards containing 0, 1.12, 2.24, 3.36, 5.6, 7.84, or 11.2 mg L^{-1} PA-P were prepared from sodium phytate (Sigma, St. Louis, MO), the P content of which was determined by ICP as 18.38%. Absorbance of color reaction products for both samples and standards was read at 500 nm on a DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA), and calculation of sample PA-P content followed the method described in Latta and Eskin (1980).

Anion Exchange Column Method

For the AEC method (Harland and Oberleas, 1986), 1 mL of the seed crude extract without NaCl treatment was diluted by mixing with 23 mL of dd H_2O and 1 mL of 0.11 mmol mL^{-1}

Na_2EDTA + 0.75 mmol mL^{-1} NaOH. Ten mL of the diluted sample was applied to an exchange column (0.7 × 15 cm) packed with anion exchange resin (AG1-X4, 100–200 mesh, Cl^- form), which was preconditioned with 1 mmol mL^{-1} NaCl to ensure chloride ion saturation. Before eluting through the column, the sample solution was held for 10 min and eluted subsequently with a total of 15 mL (3 × 5 mL) of dd H_2O, 15 mL (3 × 5 mL) of 0.1 mmol mL^{-1} NaCl, and 15 mL (3 × 5 mL) of 0.7 mmol mL^{-1} NaCl. Elutes obtained in different fractions were collected separately with 15-mL plastic tubes and analyzed by ICP for P content. Phosphorus eluted in 0.7- mmol mL^{-1} NaCl is considered PA-P, and in the rest of the fractions was considered Pi.

High-Performance Liquid Chromatography Analysis

A 0.5-g sample of soybean powder was mixed with 10 mL of 0.5 mmol mL^{-1} HCl and stirred for 1 h in a 20-mL vial. Crude extract was centrifuged at 18,000 *g* for 10 min in a microcentrifuge. An aliquot of 1 mL of supernatant was filtered through a 13-mm/0.45-μm syringe filter using a 1-mL tuberculin syringe. Chromatography of each sample was performed on a binary HPLC system equipped with a 7.5- × 4.6-mm guard column. Eluted PA was reacted with Wade reagent (0.015% $FeCl_3 \cdot 6H_2O$ + 0.15% sulfosalicylic acid) in a postcolumn tube. The absorbance of samples' color reagent products at 500 nm was processed and integrated by the chromatographic data acquisition system (Kwanyuen and Burton, 2005).

Solution ^{31}P Nuclear Magnetic Resonance Analysis

In a 1.5-mL microcentrifuge tube, ^{31}P NMR analysis samples were prepared by mixing 450 μL of acid extracts (NaCl-treated supernatants) with 450 μL of 0.11 mmol mL^{-1} Na_2EDTA + 0.75 mmol mL^{-1} NaOH, 40 mg NaOH powder, and 100 μL D_2O, which was added for signal locking. The solution ^{31}P NMR spectra were acquired on a Varian Unity 400 MHz spectrometer (Walnut Creek, CA) with an automated ^{31}P probe robust operating at 161.9 MHz. A 70° pulse was used with 0.819 s acquisition time, 3 s delay, and broadband proton decoupling. A total of 1000 scans were collected for each sample, and 85% phosphoric acid was used as an external standard. Spectra were integrated with NMR Utility Transform Software (NUTS) for Windows (Acron MR, Inc., Livermore, CA). The signal assignments for various P components were orthophosphate (Pi = 6.28 ppm), PA (PA = 4.54, 4.70, 5.06, and 5.95 ppm), and other organic P (4.00–6.00 ppm other than PA signals) (Turner, 2004). Phytic acid was quantified by its C_2–P chemical shift (5.95 ppm), which was isolated from the rest of monoester peaks. Quantification was achieved by multiplying the atomic percentage (peak area percentage) of each type of P with total ICP-determined extracted P.

Statistical Analysis

Minitab 14 (Minitab, 2004) was used for all statistical and graphical analysis except for analysis of covariance (ANCOVA). SAS version 9.1 (SAS Institute, 2003) was used in ANCOVA to examine the differences in PA across the four assay methods, using Pi determined by the AEC method as a covariate.

RESULTS AND DISCUSSION

Modifications of the Colorimetric Method

The colorimetric method of Vaintraub and Lapteva (1988) was found to have poor precision and reproducibility. It also underestimated the PA content when crude soybean seed extracts were used in our study (data not shown), which is in agreement with other reports (Fruhbeck et al., 1995). The method apparently suffered from severe matrix interference with unpurified extracts, although the nature of the interference is unknown. In the earlier development of the AEC method, a NaOH + EDTA solution was used successfully to remove unwanted matrix effects (Harland and Oberleas, 1986). However, EDTA cannot be applied in this colorimetric assay because it interferes with the reaction of the Wade reagent (Tze-Hung Yang, personal communication, 2006). We tested high-speed centrifugation at 11,000 *g* and precipitating crude soybean extracts in 10% NaCl solution at 4°C for 60 min followed by low-speed centrifugation at 1000 *g*. The NaCl cleaning step proved to be more effective than a single-step high-speed centrifugation for removing the matrix interference.

The reproducibility and precision of the modified colorimetric method was confirmed through numerous independent assays of the PA-P content of the reference sample, CX1834-1-6. An *F* test of 10 different runs showed that repeated determinations of CX1834-1-6 PA-P content did not vary significantly ($P > 0.38$) from one run to another. The CV of five replications within each run was between 1.8 and 4.2% for the 10 independent runs, indicating that the use of NaCl cleaning resulted in an improved analytical precision and reproducibility. However, the centrifugation at 11,000 *g* did not produce the same precision and reproducibility as the NaCl treatment, suggesting that a much higher centrifugation speed is needed. The NaCl-treated soybean extracts were stable at 4°C up to 24 h. Our modifications are important for those who do not have access to ultra-high-speed centrifugation. With our modified protocol, more than 200 samples can be processed every 2 d, with the only cost being the Wade reagent. The protocol can be converted to a higher throughput procedure using a 96-well microtiter plate reader and single soybean seed samples.

Comparison of the Modified Colorimetric Method with Three Other Methods

Phytic acid P content determined by the modified colorimetric method was highly correlated ($P < 0.01$) with measurements by the three well-established methods, HPLC, AEC, and ^{31}P NMR (Table 1, Fig. 1). Above 3.0 mg P g^{-1} level, colorimetric PA-P values were close to those determined by AEC, HPLC, and ^{31}P NMR, except for a few cases measured by ^{31}P NMR (Fig. 1). Below 2.5 mg P g^{-1}, the modified colorimetric method detected PA-P values consistently higher than HPLC and ^{31}P NMR.

Table 1. Correlation coefficients between phytic acid P determined by the modified colorimetric, high-performance liquid chromatography (HPLC), anion exchange column (AEC), and nuclear magnetic resonance (NMR) methods.

Method	Colorimetric	HPLC	AEC	^{31}P NMR-1†
HPLC	0.96**			
AEC	0.97**	0.99**		
^{31}P NMR-1†	0.94**	0.96**	0.96**	
^{31}P NMR-2†	0.92**	0.98**	0.97**	0.95**

**Significant at the 0.01 probability level.

†^{31}P NMR-1 = phytic acid P (IP_6) by NMR; ^{31}P NMR-2 = phytic acid P (IP_6) and other inositol phosphates (IP_1, IP_2, IP_3, IP_4, and IP_5) by NMR.

The modified colorimetric method overestimated the PA-P content for low PA samples. A possible explanation for this overestimation was that samples with PA-P less than 2.5 mg P g^{-1} contain higher Pi, which, like PA, precipitate with Fe^{3+} in the Wade reagent and interfere with the colorimetric analysis.

A comparison of the mean and range of seed PA-P content for the 42 soybean lines determined by the four methods is shown in Fig. 2. The modified colorimetric method had the narrowest range with a minimum of 1.91 mg g^{-1}, a maximum of 6.20 mg g^{-1}, and a slightly higher mean of PA-P (3.51 mg g^{-1}), than the other three methods. The higher minimum value could have been due to the Pi interference in the colorimetric analysis. The HPLC and AEC methods can remove Pi interference by species separation, and the ^{31}P-NMR method can remove Pi interference by spectroscopic separation because Pi has its distinctive chemical shift. The mean values by HPLC and ^{31}P NMR were almost the same when other inositol phosphate species (IP_1, IP_2, IP_3, IP_4, and IP_5) were not included, but the ^{31}P NMR PA-P had a wider distribution. The AEC gave a higher mean value than HPLC and ^{31}P NMR-1, which may be because the 0.7 mmol mL^{-1} NaCl eluent of AEC may have contained other inositol phosphates (IP_1, IP_2, IP_3, IP_4, and IP_5). To confirm this, we recalculated the PA-P contents of samples for ^{31}P NMR method by including other inositol phosphates. The data distribution (^{31}P NMR-2) was similar to that of AEC (Fig. 2), although the ^{31}P NMR-2 gave a higher mean value. The HPLC and ^{31}P-NMR methods gave more accurate PA-P analysis than either the modified colorimetric or AEC method because neither can separate other inositol phosphates from IP_6.

Table 2. Parameter estimates and *t* tests for the analysis of covariance (ANCOVA) model, $Y_{ij} = \beta_0 + \sum_{j=1}^{3}\beta_j Z_j + \beta_4 X_i + \sum_{j=1}^{3}\beta_{j+4} X_i Z_j + \varepsilon_{ij}$, where Y_{ij} = detected PA-P (mg g^{-1}) from the *i*th sample (i = 1, 2, …, 42) obtained from the *j*th PA assay method; and j = 1, 2, 3, where j = 1 corresponds to high-performance liquid chromatography (HPLC), j = 2 corresponds to anion exchange column (AEC), and j = 3 corresponds to ^{31}P nuclear magnetic resonance (NMR).

Parameter name	Parameter	Estimate	*t* statistic	*P* value
Colorimetric intercept	β_0	4.829	20.79	0.0000
HPLC intercept	β_1	0.270	0.82	0.4124
AEC intercept	β_2	0.656	2.00	0.0476
^{31}P NMR intercept	β_3	0.441	1.34	0.1812
Colorimetric slope	β_4	−0.545	−6.52	0.0000
HPLC slope	β_5	−0.306	−2.59	0.0106
AEC slope	β_6	−0.347	−2.93	0.0039
^{31}P NMR slope	β_7	−0.388	−3.28	0.0013

To explore the differences between the four PA assay methods in determining the PA content as Pi content varies among the 42 soybean samples, the full ANCOVA was specified as follows:

$$Y_{ij} = \beta_0 + \sum_{j=1}^{3}\beta_j Z_j + \beta_4 X_i + \sum_{j=1}^{3}\beta_{j+4} X_i Z_j + \varepsilon_{ij}$$

where Y_{ij} = detected PA-P (mg g^{-1}) from the ith sample (i = 1, 2, …, 42) obtained from the jth PA assay method; j = 1, 2, 3 corresponds to the HPLC, AEC, and ^{31}P NMR methods; β_0 = modified colorimetric method intercept and β_4 = modified colorimetric method slope; X_i = inorganic P (mg g^{-1}) content in a given sample; Z_j = 1, when PA assay method j was used; otherwise Z_j = 0; and ε_{ij} = random error.

The use of the indicator variables (Z_j) in the full ANCOVA model allows for the possibility of four distinct linear regression models describing the relationship between PA and Pi. Testing for coincidence between the four models using partial F tests (H_0: $\beta_1 = \beta_2 = \beta_3 = \beta_5 = \beta_6 = \beta_7 = 0$) was rejected ($F = 4.46$, $P < 0.01$). Testing for the intercept coefficients (H_0: $\beta_1 = \beta_2 = \beta_3$) failed to produce significant differences ($F = 1.42$, $P > 0.2$), whereas testing for interaction between Pi and the assay methods used (H_0: $\beta_5 = \beta_6 = \beta_7 = 0$) gave highly significant differences ($F = 4.46$, $P < 0.01$). The four assay methods resulted in the same value of PA-P when Pi was approximately 0 mg g^{-1}. But when Pi increased up to 5 mg g^{-1}, the assays significantly differed from each other in the PA-P for a given amount of Pi present in the sample.

Interaction between Pi and assay methods was further analyzed by comparing the HPLC, AEC, and ^{31}P NMR against the modified colorimetric assay. For the intercept coefficients β_0, β_1, β_2, and β_3, t tests showed at the $\alpha = 0.05$ level no significant differences either between HPLC and the modified colorimetric method ($P > 0.4$) or between ^{31}P NMR and the modified colorimetric method ($P > 0.18$) (Table 2). For the slope coefficients β_5, β_6, β_7, t tests showed significant ($P < 0.01$) interaction between Pi content and PA assay methods. At the $\alpha = 0.01$ level, PA-P content can be estimated by the seed Pi in a soybean line with the following linear regression models:

$$\text{Phytic acid P}_{\text{by Colorimetric}} = 4.829 - 0.545\ \text{Pi}$$

$$\text{Phytic acid P}_{\text{by HPLC}} = 4.829 - 0.851\ \text{Pi}$$

$$\text{Phytic acid P}_{\text{by AEC}} = 5.485 - 0.892\ \text{Pi}$$

$$\text{Phytic acid P}_{\text{by NMR}} = 4.829 - 0.932\ \text{Pi}$$

Although these models were developed based on only 42 samples, it is possible to estimate PA-P content accurately based on the Pi content in a given sample by developing prediction models in a similar fashion as presented here and using a large set of samples. The models developed in this study include both intercept and slope coefficients accounting for total P variation and are more accurate than the simple inverse estimation of PA-P by Pi used in the indirect PA assay (Chen et al., 1956; Raboy et al., 2000), which considered only the PA-P/Pi ratio.

Applicability of the Modified Colorimetric Method for Breeding Low PA Soybean

The PA-P content of 42 soybean lines by the four methods was plotted against Pi content determined by the AEC method. The results indicate a general inverse relationship between Pi and PA-P; however, closer inspection shows that the relationship broke down at Pi levels below 1.5 mg P g^{-1}. For example, when Pi is about 1 mg P g^{-1}, PA-P ranged from 4 to 7 mg P g^{-1} (Fig. 3). The results show that the use of Pi content as an indicator of PA content is valid only at certain Pi levels. The assumption for using Pi as a PA indicator is an invariant total P content among samples, and the indirect Pi assay only considers the PA-P/Pi ratio, neglecting the total P variation among samples. According to our assay results, the total P contents of the 42 samples in this study ranged from a minimum of 4.78 mg P g^{-1} to a maximum of 7.75 mg P g^{-1}, with a mean of 5.91 mg P g^{-1}, and more than 25% of the samples had total PA content 15 to 25% higher or lower than the average. The correlation coefficient between Pi and PA-P was only about 0.70 to 0.88 depending on the PA assay methods used. By comparison, the modified colorimetric method was a direct and quantitative PA assay, which produced correlation coefficients of 0.94 to 0.97 results with the three well-established methods, HPLC, AEC, and ^{31}P NMR.

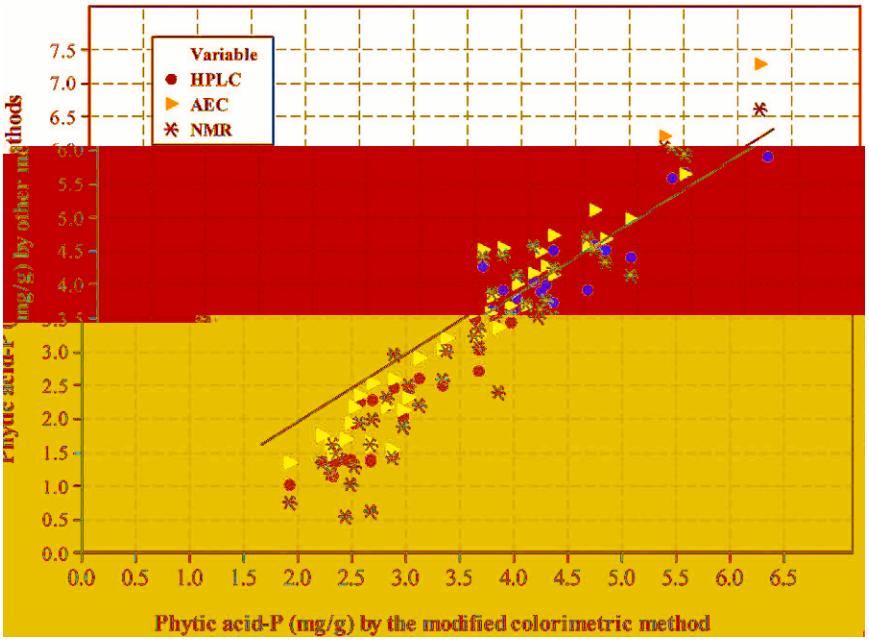

Figure 1. Correlation between seed phytic acid P content of 42 soybean genotypes determined by the modified colorimetric method and by the other assay methods: high-performance liquid chromatography (HPLC), anion exchange column (AEC), and nuclear magnetic resonance (NMR). The solid line ($y = x$) represents a 1:1 relationship between colorimetric phytic acid P and phytic acid P determined by other methods.

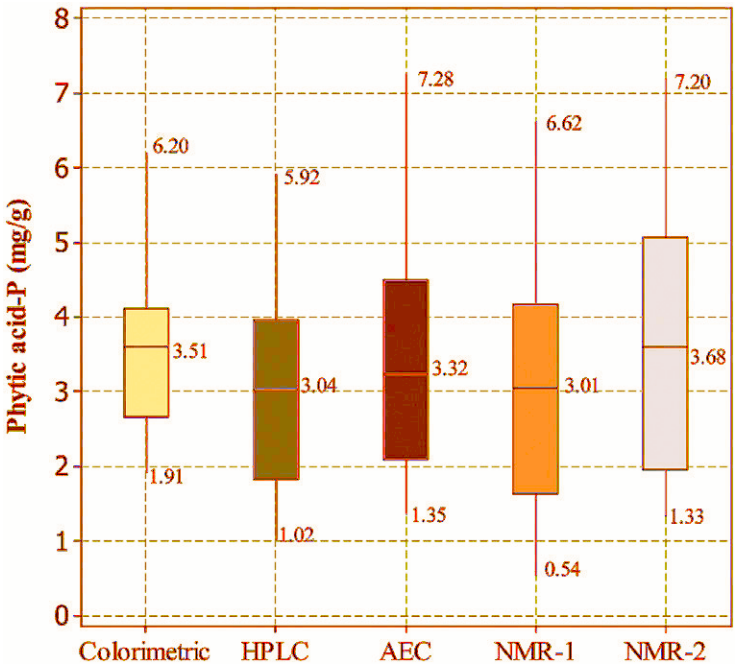

Figure 2. Mean and range of seed phytic acid P content determined by the modified colorimetric, high-performance liquid chromatography (HPLC), anion exchange column (AEC), and ^{31}P nuclear magnetic resonance (NMR) methods for 42 soybean genotypes. NMR-1 = phytic acid P (IP_6) content determined by the ^{31}P NMR method; NMR-2 = phytic acid P (IP_6) and other inositol phosphates (IP_1, IP_2, IP_3, IP_4, and IP_5) determined by the ^{31}P NMR method.

Compared with the HPLC, AEC, and ^{31}P-NMR methods, the modified colorimetric method is simpler, less expensive, and more practical and useful for screening a large number of soybean samples. Figure 4 shows the correspondence of PA-P levels of 11 soybean genotypes as determined by the modified colorimetric method as well as by the HPLC, AEC, and ^{31}P-NMR methods. The four methods basically give the same rank order for the different soybean genotypes as 1272 (a Virginia Tech breeding line developed from CX1834-1-6 × V99-3337) < CX1834-1-6 < V99-5089 < M766 ≤ Hutcheson ≤ V99-3337 ≤ V99-8060

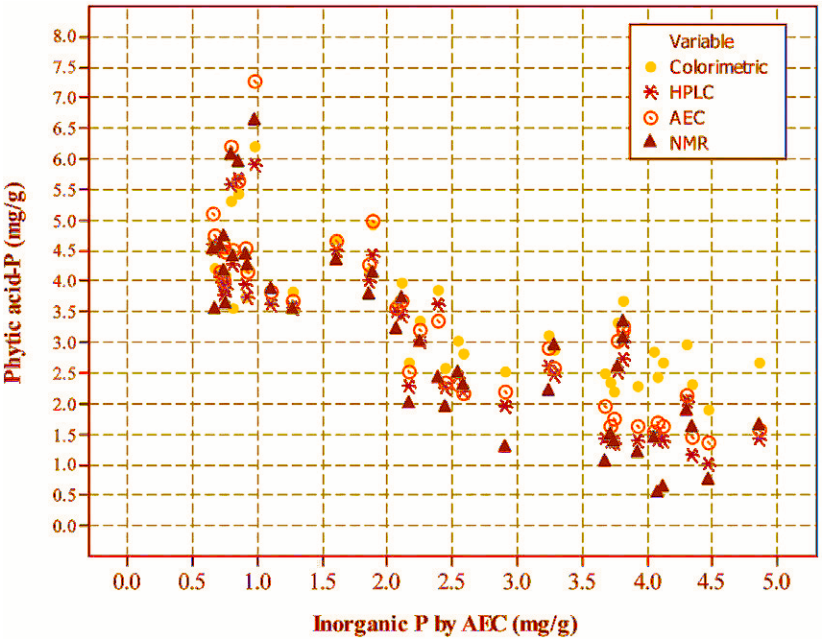

Figure 3. Scatterplot of seed inorganic P (Pi) content of the 42 soybean genotypes measured by the anion exchange column (AEC) method corresponding to their phytic acid P content as measured by different methods, colorimetric, high-performance liquid chromatography (HPLC), and nuclear magnetic resonance (NMR).

≤ PI 87013 ≤ MN 1401 ≤ MFL 552 < PI 407162, indicating the effectiveness of the modified colorimetric method for screening purposes.

The modified colorimetric method overestimated the PA-P content in soybean lines containing high Pi content. The Pi level thresholds for high Pi soybean lines are identified in Fig. 5. Below these Pi thresholds, the differences in the analyzed PA-P content by the four methods were less than 1 mg g^{-1}. When Pi ≤ 3.27 mg g^{-1}, HPLC and the modified colorimetric determined PA-P with less than 1 mg g^{-1} difference. When Pi ≤ 4.78 mg g^{-1}, AEC and the modified colorimetric method determined PA-P with less than 1 mg g^{-1} difference, and when Pi ≤ 2.59 mg g^{-1}, ^{31}P NMR and the modified colorimetric determined PA-P with less than 1 mg g^{-1} difference.

With the modified colorimetric method, thousands of soybean breeding lines have been screened for low PA content in the breeding program at Virginia Tech. Based on the PA content, pedigree selections and progeny tests in our breeding program have been efficient and effective. The *pha1* and *pha2* (Oltmans et al., 2004) associated with high Pi level in mapping populations derived from CX1834-1-2 (Walker et al., 2006) also were confirmed to be highly associated with low PA content as determined by the modified colorimetric method in our quantitative trait loci (QTL) mapping population derived from CX1834-1-6, a sister line of CX1834-1-2 (Gao et al., 2006).

Acknowledgments

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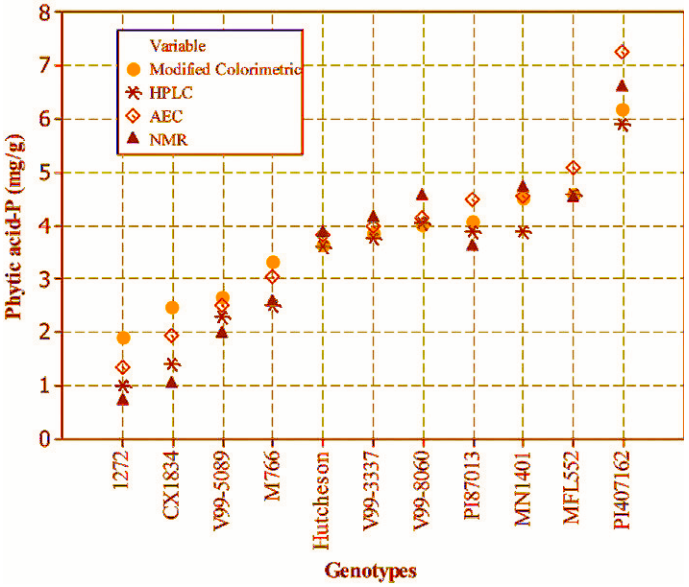

Figure 4. Ranking of phytic acid P content among 11 soybean genotypes with the modified colorimetric, high-performance liquid chromatography (HPLC), anion exchange column (AEC), and ^{31}P nuclear magnetic resonance (NMR) methods.

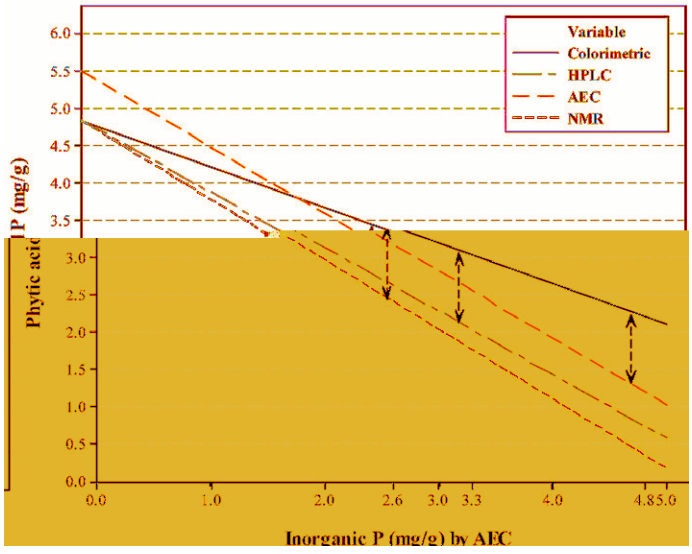

Figure 5. Linear regression relationship between anion exchange column (AEC)–inorganic P and phytic acid P associated with each analytical method. The arrows represent a difference of 1 mg g^{-1} phytic acid P between the indicated assay methods.

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